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Genes involved in strigolactone biosyntheses and their expression analyses in columnar apple and standard apple

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Abstract

Columnar apple is a valuable resource for genetic improvement of cultivated apples due to its special tree architecture. Strigolactones (SLs) are a novel class of plant hormones controlling shoot branching. The content of SLs is higher in columnar apple than in standard apples. In this study, the members of major gene families involved in SLs biosynthesis and signaling were identified from apple genomic sequences and their expression profiles were characterized in columnar and standard apples using reverse transcription quantitative polymerase chain reactions. In comparison with standard apple, the higher expressions of *MORE AXILLARY GROWTH* genes *MdMAX3-1* and *MdMAX4-4* were detected in both buds and shoots of columnar apple but the expression of *DWARF* gene *MdD53-4* showed a lower expression in columnar apple. Overexpression of Columnar gene *MdCo31* in tobacco increased SLs content and weakened the inhibition of SLs signal transduction by increasing expression of *MAX3* and down-regulating the transcription of *D53*. Thus *MdCo31* could be a strong candidate gene for the control of columnar habit.

Additional key words: Columnar gene; *Malus × domestica*, *Nicotiana tabacum*, shoot branching, tobacco.

Introduction

The plant architecture is regulated by genetic factors, phytohormones, and environment factors as well. Phytohormones play a critical role in regulating shoot branching (Domagalska and Leyser 2011, Hollender and Dardick 2014). Several studies with branching mutants in some plant species have demonstrated that strigolactones (SLs) are the repressing hormone of long-shoot branching in both monocots and eudicots (Gomez-Roldan *et al.* 2008, Umehara *et al.* 2008, Zhou *et al.* 2013). Many naturally biosynthesized SLs have been identified and all share similar chemical structures, and are synthesized through the carotenoid cleavage pathway (Matusova *et al.* 2005, Kim *et al.* 2010, Yue *et al.* 2015). The SLs are categorized as apocarotenoids, and β -carotene is their substrate for synthesis (Al-Babili and Bouwmeester 2015).

Several studies have shown that *DWARF3* (*D3*), *D10*, *D14*, *D17*(*HTD1*), and *D27* in rice (Zou *et al.* 2006, Arite

et al. 2007, 2009, Lin *et al.* 2009), *MORE AXILLARY GROWTH 1* (*MAX1*), *MAX2*, *MAX3* and *MAX4* in *Arabidopsis* (Stirnberg *et al.* 2002, Sorefan *et al.* 2003, Booker *et al.* 2004), *RAMOSUS 1* (*RMS1*), *RMS4* and *RMS5* in pea (Beveridge 2000), *DECREASED APICAL DOMINANCE 1* (*DAD1*), *DAD2* and *DAD3* in petunia (Snowden *et al.* 2005, Hamiaux *et al.* 2012) are involved in either the biosynthesis or signaling of SLs (Zhou *et al.* 2013). Among these genes, *MAX3/RMS5/D17/DAD3*, *MAX4/RMS1/D10/DAD1*, *MAX1* in *Arabidopsis* and *D27* in rice encode the carotenoid cleavage dioxygenase (CCD7), CCD8, CYP711A1 (a cytochrome P₄₅₀) and a novel β -carotene isomerase, respectively, and they are involved in the sequential cleavage of β -carotene and synthesis of SLs (Alder *et al.* 2012). In contrast, *MAX2/RMS4/D3* and *D14/DAD2*, which encode an F-box protein and a protein of the α/β -hydrolase superfamily, respectively, likely play a role in SLs signaling (Stirnberg *et al.* 2007, Arite *et al.* 2009, Hamiaux *et al.* 2012). The

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Abbreviations: CCD - carotenoid cleavage dioxygenase; Co - *COLUMNAR*; D - *DWARF*; EST - expressed sequence tags; MAX - *MORE AXILLARY GROWTH*; RT-qPCR - reverse transcription quantitative PCR; SL - strigolactone; SPAD - chlorophyll content.

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mutation of gene at stage of SLs biosynthesis could affect the whole synthesis process, and ultimately affect the regulation of the branching. *MAX3* and *MAX4* encode two enzymes known as CCD7 and CCD8. The mutants *max3* and *max4* have more branched phenotypes compared to wild-type (Arite *et al.* 2007, Drummond *et al.* 2000, Steo *et al.* 2014). The *MAX1* is required for the synthesis of active SLs. The enzyme activity of *MAX1* was originally inferred from grafting experiments when *max1* mutant rootstocks grafted to *max3* or *max4* mutant scions have wild-type levels of branching whereas reciprocal grafts are highly branched (Booker *et al.* 2005; Lazar *et al.* 2006). That result suggests the existence of a transmissible signal, which is downstream of the enzymes described above. The *MAX3* and *MAX4* could control the biosynthesis of the same graft-transmissible signal, but they have to act in the same tissue. On the other hand, *max1* and *max3* or *max4* double mutant phenotype have no additional effect, indicating that *max1* stock can produce a transmissible signal substance that is missing in *max3* and *max4* (Booker *et al.* 2005, Lazar *et al.* 2006). The results indicated that *MAX1* have actions in the downstream of *MAX3* and *MAX4*. The DAWARF53 (D53) was the first target protein of the D14-SCFMAX2 complex and its degradation led to the repression of lateral shoot growth (Zhou *et al.* 2013).

The columnar apple was initially discovered in ‘Wijcik McIntosh’, a sort of ‘McIntosh’ (Kesley and Brown 1992). This columnar habit is characterized by short internodes, reduced long branches, and increased lateral spurs on branches. The columnar habit could be attributed to the dominant allele of a single gene, *Columnar* (*Co*) (Petersen and Krost 2013, Hollender and Dardick 2014). To identify the *Co* gene and develop DNA markers for the columnar growth phenotype, apple genetic linkage maps have been constructed, which showed that the *Co* gene is located on linkage group 10 (Moriya *et al.* 2009). Several researches have reported the fine mapping of the *Co* gene and mutations of the *Co* region (Bai *et al.* 2012, Moriya *et al.* 2012, Zhang *et al.* 2012, Baldi *et al.* 2013, Wolters *et al.* 2013, Otto *et al.* 2014, Petersen *et al.* 2015, Okada *et al.* 2016). Recent studies with comparing the genomic sequence of the *Co* region of columnar with standard apples have demonstrated that a DNA element/Gypsy retrotransposon is inserted in an intergenic region of columnar apple (Wolters *et al.* 2013, Otto *et al.* 2014, Okada *et al.* 2016). Expression analysis of selected genes located in the vicinity of insertion revealed the upregulation of *MdCo31* gene, encoding a putative 2OG-Fe(II) oxygenase or downy mildew resistant 6 (*DMR6*) (Wolters *et al.* 2013, Otto *et al.* 2014). Overexpression of *MdCo31* in *Arabidopsis thaliana* and apples resulted in phenotypes with short internodes (Wolters *et al.* 2013, Otto *et al.* 2014, Okada *et al.* 2016). These results implied that *MdCo31* could be a strong candidate gene for the control of columnar habit.

In order to further study the relationship between SLs and columnar traits, 23 SLs biosynthesis and signaling related genes were identified in apple genome data following *TBLASTN* with amino acid sequences from *Arabidopsis*, pea, rice, and petunia based on previous

reports. These genes included five *MdMAX1*-related genes, one *MdMAX2*, two *MdMAX3*, three *MdMAX4*, five *MdD14*, three *MdD27*, and four *MdD53*. The transcription patterns of twenty-three SLs biosynthesis and signaling related genes were detected using real-time PCR from different developmental buds and shoots of columnar and standard apple trees.

Materials and methods

Plants: The plant materials comprised four standard apples (*Malus × domestica* Borkh. cv. McIntosh, genotypes 21-S, 31-S, and 77-S) and four columnar apples (cv. Wijcik, genotypes 21-C, 31-C, and 77-C which were natural standard mutants of 21-C, 31-C, 77-C, respectively). Those trees were maintained in the Jiaozhou farm of Qingdao Agricultural University. Buds begin to germinate (0-d-old buds), 30-d-old buds, 60-d-old buds, as well as new shoots 30-d-old, 60-d-old, 90-d-old, and 120-d-old were collected from each sampling tree in the field and kept in ice box for about 1 h during transport, and then put into liquid nitrogen or ultralow temperature refrigerator.

Gene predication and expressed sequence tags (EST) analysis: Scaffold sequences of SLs biosynthesis/signaling related genes in several species were identified from the non-redundant gene list (<http://www.rosaceae.org/node/58>) of apple draft genome sequences through a *TBLASTN* search (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>). Gene models were predicted from scaffold sequences using the *GENSCAN* web server (<http://genes.mit.edu/GENSCAN.html>).

The protein sequences of candidate genes in apple were used as queries to search the strigolactone biosynthesis related-EST sequences in each plant EST database (<https://www.ncbi.nlm.nih.gov/dbEST/index.html>). The alignment parameters were: score >100 and E-value >1×10^{−20}. The tissue types of selected ESTs were isolated from the *EST-GenBank* format file, which was downloaded from *NCBI dbEST*. Target sequences were blasted back against *Arabidopsis thaliana* annotated sequences to obtain correct sequences.

Transcription profiling by reverse transcription quantitative PCR (RT-qPCR): Total RNA from each sample was prepared using a *RNAprep* pure plant kit according to the manufacturer’s instructions (Tiangen Biotech, Beijing, China). First-strand cDNA was synthesized from 2 µg of the total RNA using a *HiScriptII* 1st strand cDNA synthesis kit (Vazyme, Nanjing, China) and poly dT as a primer. The RT-qPCR was performed in three technical replicates for each of the three biological samples using a *ChamQTM Universal SYBR qPCR* master mix, 10 ng of cDNA, and 0.2 µM each primer according to the manufacturer’s instructions (Vazyme). The *MdActin*, stably expressed in all of the tissues, was selected as a reference gene (Zhang *et al.* 2018). Relative expressions were calculated using the comparative Ct method with normalization of data to the geometric average of the

reference gene expressions by the equation $2^{-\Delta\Delta Ct}$. The primers used in RT-qPCR are summarized in Table 1 Suppl.

Strigolactone quantification: The SLs extraction and quantification by ultra performance liquid chromatography/mass spectrometry from buds were carried out as described by Zhou *et al.* (2013). Known amounts of deuterium-labeled standards for orobanchol, orobanchyl acetate, and fabacyl acetate (Zhou *et al.* 2013) were added before extraction, and endogenous hormone content was calculated from the ratio of endogenous to standard peak areas per gram of root fresh mass.

Vector construction for transformation of tobacco: For producing the *35S-MdCo3l*, an *EcoR I* - *Sac I* fragment containing the *MdCo3l* cDNA was cloned into the *pCAMBIA1300* binary vector, in which transgene expression was under the control of the CaMV 35S promoter. The *pCAMBIA1300-MdCo3l* was used to transform tobacco. After the construct was transferred to *Agrobacterium tumefaciens* strain EHA105a by the freeze-thaw method, this *A. tumefaciens* was used to transform the leaves of five-leaf stage tobacco (*Nicotiana tabacum* L.) seedlings (Li *et al.* 2016). Putative transgenic plants (T0) were regenerated in the presence of 40 mg dm⁻³ hygromycin B and further screened by PCR. The seeds from T0 plants were sowed on hygromycin-containing medium for selecting T1 transgenic lines.

Chlorophyll meter reading: A chlorophyll meter (SPAD-502, Minolta, Tokyo, Japan) was used to obtain leaf chlorophyll content (a SPAD value). Ten transgenic and wild-type plants were selected and SPAD values were recorded from the third (from top of the main stem) fully expanded leaf. Three SPAD value readings were taken per plants.

Statistical analysis: Values are shown as means $\pm$ SEs of three independent experiments with three replicates each. Differences among samples were analyzed by one-way analysis of variance combined with the Duncan multiple range test.

Results

Recent studies demonstrated that *MdCo3l* is a strong candidate gene for columnar habit. In order to understand the relationship between the columnar type and the *MdCo3l*, the expression patterns of *MdCo3l* were detected using real-time PCR from buds and shoots of different developmental stages of columnar and standard apples. The expression of *MdCo3l* was significantly higher in the buds and shoots of columnar apple than that in those of standard apple (Fig. 1). The *MdCo3l* was generally more transcribed in buds compared to shoots in both standard and columnar apple trees.

Based on previous reports (Stirnberg *et al.* 2002, 2007, Arite *et al.* 2007; Gomez-Roldan *et al.* 2008, Umehara

et al. 2008, Drummond *et al.* 2009, Kretzschmar *et al.* 2012, Waters *et al.* 2012, , Foo and Reid 2013, Jiang *et al.* 2013, Zhou *et al.* 2013), the strigolactones biosynthesis and signaling related genes were identified through a genome-wide strategy. Twenty-three SLs biosynthesis and signaling related genes were identified from apple genome data following *TBLASTN* with amino acid sequences from *Arabidopsis*, pea, rice, and petunia. These genes included five *MdMAX1*-related genes, one *MdMAX2*, two *MdMAX3*, three *MdMAX4*, five *MdD14*, three *MdD27*, and four *MdD53* (Table 2 Suppl., Fig. 2).

Strigolactones are widely recognized as shoot branching regulators. The SLs content was higher in columnar apple (0.34 ng g⁻¹), whereas it was 0.11 ng g⁻¹ in 'McIntosh'. To determine the expressions of all identified genes and if their expression patterns correlated with SLs biosynthesis, signaling, and the habit, expression analysis was employed in a set of buds and shoots of columnar and standard apple.

In this study, five *MdMAX* genes were identified in apple and expressions of three of *MdMAX1* (*MdMAX1-1*, *MdMAX1-2* and *MdMAX1-4*) were confirmed in buds and shoots (Fig. 1 Suppl.). The *MdMAX1-1* expression in buds and shoots of columnar apple was higher than in standard apple. The expression of *MdMAX1-4* was not detected in both columnar apple and standard apple. Only one *MdMAX2* gene was identified in apple genome. The expression of *MdMAX2* in the shoots of columnar apple was higher than in standard apple, and there was no significant difference between columnar and standard apple in its expression in buds (Fig. 1 Suppl.). Two *MdMAX3* genes were identified. The expression of *MdMAX3-1* in columnar apple was higher than in standard apple, and the expression in standard apples except 21-S was exceptionally weak (Fig. 1 Suppl.). In the buds of columnar apple, the expression of *MdMAX3-1* started to increase and then decreased, and the expression in the shoot tips of columnar apple showed a downward trend. The transcription of *MdMAX3-2* was nonspecific in the buds of columnar and standard apples (Fig. 1 Suppl.).

A total of five *D14* genes were identified. The expression of four *MdD14* genes could be detected in columnar and standard apples. The *MdD14-4* exhibited a down-regulated expression pattern in the buds whereas it demonstrated an up-regulated pattern in the shoots (Fig. 1 Suppl.). The transcript profiles of both *MdD14-3* and *MdD14-5* genes showed opposite trends (Fig. 1 Suppl.). The expression level of *MdD14-1* was lower in early columnar apple buds and shoots than in those of standard apple, but the *MdD14-5* expression of columnar apples was higher than that of standard apples (Fig. 1 Suppl.). The *D27* might also be a key gene involved in the synthesis of SLs. The *MdD27-1* expression was much lower in standard apple than in columnar apple. Buds had a comparable transcript abundance of *MdD27-1*, but a lower expression in the shoot tips (Fig. 1 Suppl.). The *D53* acted as a repressor of SL signaling. The SLs induced *D53* degradation by the proteasome and abrogated its activity in promoting axillary bud growth. The expression of three members of *MdD53* was detected. An *MdD53-*

l expression was highest in the buds and lowest in the shoot tips. The expressions of *MdD53-4* were comparable between columnar and standard apples, but *MdD53-1* and *MdD53-3* had much higher transcript abundances in standard apple than in columnar apple. Interestingly, the *MdD53-3* had comparable transcript levels in 'McIntosh' and 'Wijcik' (Fig. 1 Suppl.).

The *MdCo31* was thought to be an important gene for regulation of columnar traits. In order to characterize the role of *MdCo31* in SLs biosynthesis, signaling, and plant development, we chose to use the overexpression of *MdCo31* in tobacco plants. The *MdCo31* transgenic plants were characterized by a decreased height and a significantly shorter internode length (Table 1, Fig. 3A). The leaves become thick and chlorophyll content increased (Table 1). Although there were no significant changes in the number of branches of transgenic plants, almost

no long branch was observed in the transgenic plants (Fig. 3A). In order to investigate the function of *MdCo31* on the synthesis and signal transduction related genes of SLs, the expression of genes related to SLs biosynthesis and signaling in transgenic tobacco plants were studied (Fig. 3C). The results show that *NbMAX3* transcription was upregulated in transgenic tobacco, and *NbD53* was down-regulated. The expression of *NbMAX1* decreased in transgenic lines 8 and 9, but increased in transgenic line 10. There was no significant difference in the expression of other genes between transgenic and wild tobaccos.

Discussion

The columnar apple is characterized by short internodes, reduced lateral branches, and increased spurs (Kelsy and

Fig. 1. The expression of *MdCo31* in standard (S) and columnar (C) apple genotypes 21, 31, and 77. 1,2,3,4,5,6,7 indicate 0-d-old buds, 30-d-old buds, 60-d-old buds, 30-d-old shoots, 60-d-old shoots, 90-d-old shoots, and 120-d-old shoots. Means $\pm$ SEs, $n = 3$, *and ** - significant differences at $P < 0.01$ and < 0.001 , respectively according to the Duncan's multiple range test.

Fig. 2. The structure of apple strigolactones related genes.

Brown 1992, Hollender and Dardick 2014). Some recent studies strongly suggested that columnar growth phenotype results from the up-regulated expression of *MdCo31*, encoding proteins 2OGD or Downy Mildew Resistant 6 (Wolters *et al.* 2013, Otto *et al.* 2014, Okada *et al.* 2016). So far it has been unclear how the columnar phenotype could be generated by *MdCo31*. The phytohormones are believed to have a pivotal role in the regulation of the plant architecture (Ferguson and Beveridge 2009, Müller and Leyser 2011, Qi *et al.* 2011, Dayan *et al.* 2012, Cheng *et al.* 2013, Krost *et al.* 2013). The SLs are thought to act as a second messenger that works with auxin to maintain apical dominance (Ferguson and Beveridge 2009, Cheng *et al.* 2013, Hollender and Dardick 2014). Mutants in biosynthesis and signaling of SLs show a high degree of bud break and branching (Shinohara *et al.* 2013), which is similar to the phenotype of the columnar apple. The role of SLs has not been well studied in trees. In this study, the

content of SLs was higher in columnar apple, indicating that SL could inhibit its lateral branching.

The SLs have tricyclic-lactone (ABC-ring) and methyl butenolide (D-ring), and they are connected through an *enol* ether bridge (Alder *et al.* 2012). Recently, the SLs biosynthetic pathway has been proposed, SLs might be synthesized *via* the carotenoid pathway (Matusova *et al.* 2005, Alder *et al.* 2012, Seto *et al.* 2014). The first branching genes cloned were *MAX* genes in model plant *Arabidopsis*. *MAX3* (*CCD7*) and *MAX4* (*CCD8*) encode two CCD which are targeted to the plastids (Domagalska and Leyser 2011, Harrison and Bugg 2014). The *MAX1* belongs to the gene family encoding cytochrome P₄₅₀, and these genes are involved in the biosynthesis of hormones (Lin *et al.* 2009). The *MAX2* is the orthologous member of the F-box leucine-rich repeat protein family. The *MAX1* has a role in signal transduction of the hormones (Ishikawa *et al.* 2005, Johnson

Fig. 3. Morphological differences in wild-type (WT) and transformed tobacco plants (L1 - L10; A). Reverse transcription quantitative PCR analysis of the *MdCo31* gene in WT and transgenic tobacco lines (B). Expression analysis of genes related to strigolactones biosynthesis and signaling (C). Means $\pm$ SEs, $n = 3$, *, ** - significant differences at $P < 0.01$ and < 0.001 , respectively, according to the Duncan's multiple range test. L8 - L10 - *MdCo31* transgenic tobacco lines.

Table 1. Comparison of some characteristics in 40-d-old wild-type (WT) and *MdCo3l* transgenic (L8, L9, and L10) tobacco plants. Means $\pm$ SEs, $n = 30$, *, ** - significant differences at $P < 0.01$ and < 0.001 , respectively according to the Duncan's multiple range test. SPAD - chlorophyll content.

	Height [cm]	Internode length [cm]	Flower number	Branch number	SPAD
WT	46.08 $\pm$ 1.63	2.32 $\pm$ 0.51	43.2 $\pm$ 9.2	14.2 $\pm$ 2.1	44.13 $\pm$ 1.4
L8	16.54 $\pm$ 1.13**	1.24 $\pm$ 0.39*	44.8 $\pm$ 8.5	13.4 $\pm$ 3.5	48.57 $\pm$ 0.9*
L9	19.73 $\pm$ 1.42**	1.06 $\pm$ 0.21**	51.4 $\pm$ 5.2*	11.9 $\pm$ 1.9*	46.23 $\pm$ 1.8*
L10	18.01 $\pm$ 0.82**	0.92 $\pm$ 0.25**	68.2 $\pm$ 9.3*	16.7 $\pm$ 5.4	48.961.1*

et al. 2006, Lin *et al.* 2009, Al-Babili and Bouweester 2015). The *DWARF14* (*D14*) encodes an α/β hydrolase, which interacts with strigolactones and MAX2 and is the possible receptor of strigolactones (Nakamura *et al.* 2013). The *DWARF53* (*D53*) is the target protein of the D14-MAX2 complex and act as a repressor of SLs signaling pathway, which can abrogate SLs activity in promoting axillary bud growth (Zhou *et al.* 2013). The *D27* encodes a protein ferritin, which is localized in the chloroplast and contains an enzyme functional region and is mainly expressed in vascular cells (Lin *et al.* 2009).

To better understand the function of SLs on the formation of column type characters, the members of major genes implicated in SLs biosynthesis and signal transduction were identified in the apple genome, and the expression profiles were characterized in columnar and standard apple trees. SLs, being proposed as branch inhibiting hormone (Shinohara *et al.* 2013, Hollender and Dardick 2014), are the carotenoid-derived compounds produced by action of *MAX3* and *MAX4*. It is likely that SLs are mainly synthesized in roots (Kohlen *et al.* 2011). Here we confirmed that apple could synthesize the SLs, and interestingly they were likely synthesized in the buds. The content of SLs in buds and the number of SL biosynthesis related EST were analyzed. (Fig 1 Suppl., Table 2 Suppl.), which indicates that the synthesis of the SLs not only existed in the roots but also in the buds. The transcriptions of SLs biosynthesis related genes in the buds were higher than in the shoots (Fig. 3). Thus, we predicted that SLs could regulate the formation of columnar type characters in the buds. Seventeen SLs synthesis and signaling relevant genes were identified, of which six genes had striking differences in expression between columnar and standard apples throughout the different period of bud and shoot development (Fig. 1 Suppl.).

The *MAX3* and *MAX4* are critical for SLs biosynthesis in plants (Gomez-Roldan *et al.* 2008). The higher expressions of *MdMAX3-1* and *MdMAX4-4* in both buds and shoots of columnar apple suggest that transcript abundance of *MAX3* and *MAX4* resulted in high SLs content (Fig. 1 Suppl.). The study of high-branching mutants in several species indicated that *D27* might also be a key gene involved in the synthesis of SLs (Lin *et al.* 2009, Waters *et al.* 2012). Given the plastid localization of *D27*, it appears likely that this enzyme acts upstream of the endoplasmic reticulum-localized *MAX1*, *MAX3*, and *MAX4* in the SL pathway (Lin *et al.* 2009, Al-Babili and Bouweester 2015). Among *MdD27* genes examined,

MdD27-1 showed a high expression in columnar apple, whereas the expression of other three *MdD27* genes fluctuated at similar levels between the buds and the shoots of columnar apple (Fig. 1 Suppl.). But, the relationship between the *D27* gene and the regulated columnar type traits need further studies. The branched phenotypes of *Arabidopsis d27* are relatively mild compared with the phenotypes of other SL biosynthetic mutants (Lin *et al.* 2009, Smith and Waters 2012, Al-Babili and Bouweester 2015) indicating the presence of another 9-*cis*- β -carotene source in these mutants. The *MdMAX1-1* had a decreased expression in the buds and the shoots, which was consistent with *MdMAX3-1* and *MdMAX4-3* (Fig. 1 Suppl.). These results also add the evidences about the postulation that *MAX1* acts in a biosynthetic step downstream of *MAX3* and *MAX4* (Booker *et al.* 2005, Al-Babili and Bouweester 2015). The *max1* rootstock could restore a wild type branching pattern in *max3* and *max4* shoots, suggesting that *MdMAX1* could regulate a columnar habit (Booker *et al.* 2005, Scaffidi *et al.* 2013, Seto *et al.* 2014). The *D53*, which is an inhibitor of the SLs signaling pathway, was recently elucidated in a rice mutant (Zhou *et al.* 2013). The *MdD53-4* showed a lower expression in columnar apple (Fig. 1 Suppl.), which had a weak inhibitory effect on SLs signal transduction. In addition, the expression of *NbD53* in transgenic tobacco was lower than in the wild type (Fig. 5). The *MdD53* was likely to be central to SLs-mediated branch regulation.

The *MdCo3l* was a strongly dominant gene for regulation of columnar growth phenotype (Wolters *et al.* 2013, Otto *et al.* 2014, Okada *et al.* 2016). In our study, the higher expression in columnar apple and the heterotopic expression leading to the formation of tobacco columnar traits (Fig. 1, Fig. 3A) confirmed the role of *MdCo3l* in formation of columnar type. The expression of *NbMAX1* decreased in transgenic lines 8 and 9, whereas it increased in line 10. This result may indicate the *MdCo3l* is not a factor regulating the expression of *MAX1*. The transgenic tobacco plant exhibited an increased expression of *NbMAX3* and a decreased expression of *NbD53* (Fig. 3C). These results suggest that *MdCo3l* could increase SLs content and weaken the inhibition of SLs signal transduction by increasing expression of *MAX3* and down-regulating the transcription of *D53* and then regulate the columnar character through SLs mediated manner.

As it is commonly found for other plant hormones, SLs biosynthesis and activity could be regulated by complex networks with other hormones. This is particularly true

for auxin, abscisic acid, and brassinosteroids, which regulate SLs biosynthesis and are involved in most of the SL-regulated developmental processes (Lechat *et al.* 2012, Toh *et al.* 2012, Shinohara *et al.* 2013, Wang *et al.* 2013). The MdCo31 protein revealed the DIOX-N motif and the Fe(II)-dependent oxygenase domain. Members of the gene family containing these domains could catalyze the oxidation of organic substrates and were shown to be involved in the biosynthesis of hormones and flavonoids, and defense against downy mildew (Van Damme *et al.* 2008, Wolters *et al.* 2013, Kawai *et al.* 2014). Further study for better understanding SLs and *MdCo31* regulations of the columnar habit, in particular, how *MdCo31* and SLs interact each other, is scientifically interesting and also practically important for columnar habit management and utilization.

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